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PRINCIPAL INVESTIGATOR: James Michael Shipley, Ph.D.

CONTRACTING ORGANIZATION: Washington University
St. Louis, MO 63110

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14. **ABSTRACT**
The overall goal of this project is to generate a mouse model of the smooth muscle-related facets of tuberous sclerosis, specifically in an attempt to model the lung phenotype seen in a subset of TS patients and patients with LAM. We have conditionally targeted the TSC1 gene in smooth muscle, which results in mortality at approximately 10 weeks of age. This mouse now provides a useful model in which to investigate the function of individual MMPs or other proteins in this pathological progression, and to evaluate relevant therapeutic interventions such as rapamycin.

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Table of Contents

Introduction

The overall goal of the project is to generate a mouse model in which one of the two tumor suppressor genes responsible for tuberous sclerosis, TSC1 or TSC1, are dysregulated specifically in smooth muscle. This was to be accomplished in one of two ways, including (1) specifically targeting the TSC1 gene in smooth muscle using a doxycycline-inducible transgenic system which we adapted for expression of cre recombinase in smooth muscle, or (2) by overexpression of a potentially dominant negative form of tuberin in smooth muscle. Should either of these approaches work, the goal was then to evaluate whether matrix metalloproteinase (MMP) expression was dysregulated in the mouse model or in TSC1 -/- cells derived from these mice, whether rapamycin treatment corrects this dysregulation, and whether phenotypes seen in the mice are abrogated by breeding pertinent MMP knockout alleles into the model.

Body

In the approved statement of work, individual specific aims were broken down into tasks, and these individual tasks were planned over the two year duration of the award. In the **first specific aim** (targeting the TSC1 gene in smooth muscle using conditional cre mice we have generated and evaluate resulting phenotypes), we needed to generate mice which harbor 4 separate alleles including two floxed alleles of the TSC1 gene, one SMP8-rtTA transgene "allele", and the tetO-cre allele. This was to take two breeding steps. The first step, breeding mice containing the rtTA and cre transgenes to mice which are homozygous for the floxed TSC1 allele, was completed prior to the time of award activation. Since the inception of the award, we have now completed the second breeding step and have generated mice which have all 4 necessary alleles (2 floxed TSC1 alleles, the smooth muscle rtta transgene, and the tetO-cre trangene). Our original intent was to administer doxycycline at weaning age (3 weeks) to initiate cre recombinase expression and inactivation of TSC1 in smooth muscle cells. We instead decided to maximize the chances of discovering a phenotype by beginning doxycycline administration as early as possible, by giving it continuously both to pregnant female mice and to their offspring upon weaning. We are very excited to report that inactivation of TSC1 in this context causes mortality in these mice at an average age of 10 weeks (n=19 mice as of this report date). In addition, alveolar ducts in the lungs of these mice are preferentially enlarged relative to controls (mice on doxycycline but lacking the rtta or cre transgenes)(Figure 1), and in some cases (~30%) we see nodules similar to those seen in human TS and LAM (Figure 2). Immunostaining reveals loss of TSC1 protein in cells in which active cre recombinase is expressed (Figure 3), as these cells are marked by recombination of a ROSA allele that results in ß-galactosidase activity following cre-mediated recombination (blue). Another well-characterized readout for

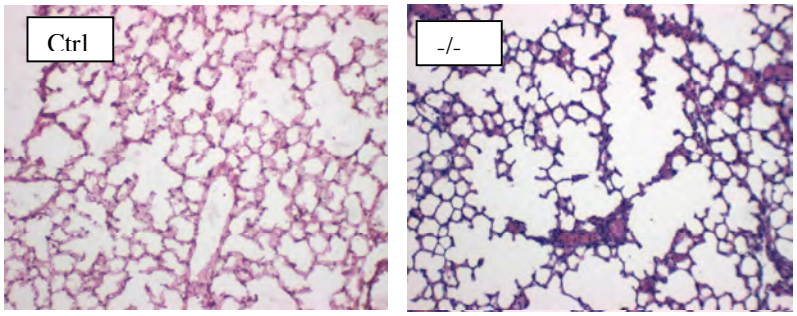

Figure 1. Morphology of TSC1 Conditional Knockout Lungs. Shown are lungs from a TSC1 conditional knockout (right) and a control littermate (left) sacrificed at 9 weeks, demonstrating enlarged airspaces in the knockout.

dysregulation of the TSC1/TSC2 pathway is activation of ribosomal protein S6 (phosphorylation), which is normally negatively regulated by the tuberin/hamartin complex. Total lung extracts from control mice show minimal phosphorylation of ribosomal protein S6(Figure 4, lanes 1-4), which is markedly induced in the lungs of our smooth muscle-specific TSC1 knockout mice (lanes 5-8). One hypothesis in TS/LAM is that lung destruction is mediated by metalloproteinases, particularly MMP-2 and -9, which is upregulated in the lungs of these patients relative to controls (Figure 5). Indeed, we observe increased MMP-9 activity in the lungs of the smooth muscle-specific TSC1 knockouts relative to controls (Figure 6). Taken together, many of the pulmonary facets of human TS/LAM are recapitulated in the lungs of the smooth muscle-specific TSC1 knockout mice.

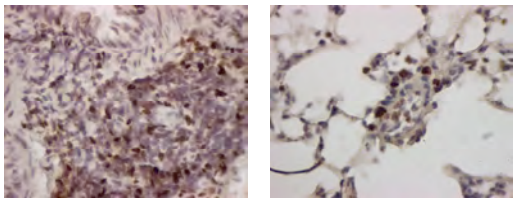

Figure 2. Ki-67 Immunostaining on TSC1 Conditional Knockout Lungs. Lungs were stained for Ki-67, a marker of proliferating cells. Areas that appear nodular show many Ki-67-positive cells.

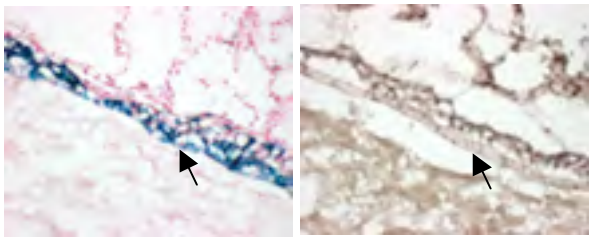

Figure 3. TSC1 immunostaining in the conditional TSC1 knockout. The ROSA26 reporter allele for cre activity was bred onto the conditional knockout background, and lung sections were stained for either ß-galactosidase activity with X-Gal (left, marks cre-positive cells) or for an antibody to TSC1 (right). Note the smooth muscle expressing cre which is negative for TSC1, in contrast to the surrounding tissue (arrows).

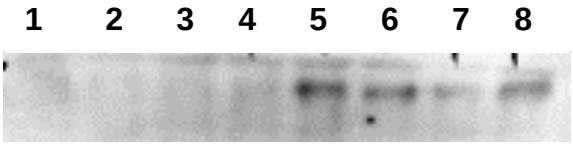

Figure 4. Activation of ribosomal protein S6 phosphorylation in TSC1 conditional knockout mice. Total lung extracts from control mice (lanes 1-4) or TSC1 conditional knockout mice (lanes 5-8) were evaluated for phosphorylation (activation) of ribosomal protein S6 by western blotting, demonstrating activation of this pathway in TSC1 conditional knockout mice.

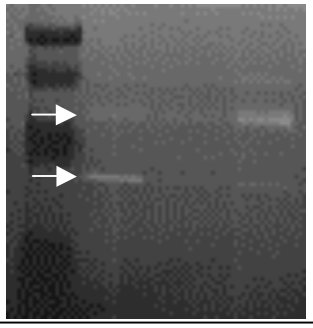

Figure 5. Expression of MMPs –2 and –9 in LAM. Ten microliters of BAL from each of three LAM patients was analyzed by gelatin zymography. Expression of MMP-9/gelatinse B (top arrow) and MMP-2/gelatinase A (bottom arrow) is seen. These proteases are undetectable in the BAL of control subjects (not shown). Increased expression of MMP-2 and MMP-9 corroborates gene expression profiling data by others on LAM tissue which shows these MMPs induced in LAM relative to biopsy specimens from control subjects.

Figure 6. Increased MMP-9 activity in Conditional TSC1 knockout lungs. The caudal lobe of the right lung of two controls (lanes 1 and 2) and one conditional TSC1 knockout (lane 3) were used for gelatin zymography. Increased MMP-9 activity is seen in the the total lung extract of the conditional TSC1 knockout animal.

The goals of the **second specific aim** were very similar to those of the first aim, but in this case the approach was to overexpress a potentially dominant negative form of the TSC2 gene product, tuberin, in smooth muscle cells in mice with the goal being to inactivate TSC function. While we were able to achieve significant overexpression of this form of tuberin in these mice upon induction by doxycycline (Figure 7), this approach did not yield a phenotype, at least after 6 months of doxycycline treatment. Thus, as our overall hope was for one of these two models to prove fruitful, and the conditional TSC1 knockout (specific aim 1) has proven to yield a highly penetrant phenotype, we have focused our attention on the analysis of the phenotype in this model.

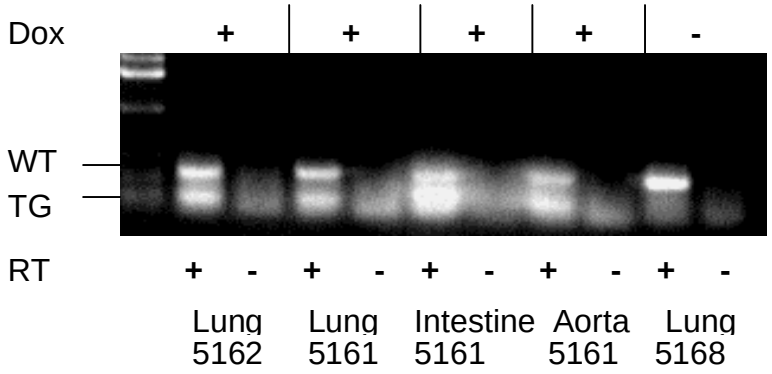

Figure 7. Induction of dominant negative TSC2 transgene expression with doxycycline. RNA was isolated from smooth muscle-containing organs from 3 transgenic mice(5161, 5162, 5168). Two (5161, 5162)had received doxycycline (Dox) in the drinking water for 2 weeks prior to sacrifice. RT-PCR for TSC2 demonstrates doxycycline-dependent induction of the transgene product (TG). Expression of the wild type TSC2 gene product is seen in all samples (WT). Either product is only seen when reverse transcriptase is used (RT), demonstrating that neither arises from DNA contamination of the RNA.

One of the goals of **specific aim 3** was to treat mice with rapamycin should they develop a phenotype, in an attempt to abrogate the development of pathological changes. At the time of grant submission, we felt that it was unlikely that this would be possible within the two year time frame of the grant. However, we have now made this a priority and are preparing for these studies. We are currently awaiting a decision from Wyeth Pharmaceuticals as to whether they will supply us with enough rapamycin to do these experiments, but based on conversations with Wyeth, we are optimistic that they will. Based on other studies in mice, we will first attempt a dose of 2-4 mg/kg IP, 3X/week. One outcome that will be monitored will be lifespan of the mice following rapamycin treatment. In addition, we will also determine whether rapamycin is able to reduce formation of pulmonary nodules, and diminish the induction of MMP expression, particularly MMP-9.

For the experiments in mice involving rapamycin, we believe it would be beneficial to increase the yield of mice which contain all 4 necessary alleles. To this end, we are in the process of modifying our breeding strategy. The goal of the new strategy will be to generate mice which are either homozygous for both the rtTA transgene and the floxed TSC1 allele, and mice which are homozygous for the cre transgene and the floxed TSC1 allele. Upon breeding mice of these genotypes, ***all*** of the offspring would contain the 4 necessary transgenes, thereby markedly increasing the efficiency of the process. Unfortunately, we have no genotyping assay that will discriminate between mice which are heterozygous vs. homozygous for the rtTA or cre transgenes. Ultimately, this test will be strictly empirically determined, ie., we will only know that a parent is homozygous for either the rtTA or cre transgenes when all of its offspring are positive for the transgene. We are currently evaluating candidates by a combination of quantitative southern blotting and real time PCR of genomic DNA for the rtTA and cre transgenes. We hope to have candidate mice within the next month that will allow us to set up these new breeding pairs in which all the offspring will carry each transgene/allele.

A number of other experiments outlined in the statement of work have been begun and are currently ongoing. These include evaluation of tissues taken from the conditional TSC1 mice by immunostaining using antibodies to TSC1 (see Figure 3), MMP-9, smooth muscle actin, and Ki-67 (a marker of proliferating cells, see Figure 2). As another approach to evaluate cell proliferation in these mice, we will do BrdU labeling and quantitate the number of BrdU positive cells per field in conditional knockouts and controls. We also recently began culturing lung fibroblasts from conditional knockouts vs. controls to gain an enriched population of adult lung cells, positive for smooth muscle actin, in which the TSC1gene has been inactivated. These cells will be used to ascertain effects on MMP expression, and to evaluate the effect of rapamycin on MMP expression in this context. We have not yet begun the similar explant experiments with aortic smooth muscle cells outlined in the statement of work, as these experiments require many more mice. In contrast, a single lobe of a mouse lung will yield a 60 mm dish of fibroblasts.

Finally, we outlined in the statement of work that should MMP induction (or repression) be seen in the TSC1 conditional knockout mice, we would attempt to breed the relevant MMP knockout allele into the model in order to evaluate the contribution of the particular MMP to the pathologic progression. Indeed, MMP-9 and –12 have been implicated as contributing to the airpsace enlargement seen in human COPD, as well as in animal models of pulmonary emphysema. As we have detected increased MMP-9 expression in the TSC1 conditional knockout (both in TSC1 -/- embryonic fibroblasts as well as in the lungs of the adult mice), it will be interesting to determine whether the lifespan or the degree of airspace enlargement is altered in the absence of MMP-9. To this end, we have begun to breed the MMP-9 knockout (which we generated several years ago) into the conditional TSC1 model. We originally anticipated that these breedings would not begin until well into the second year of funding, so these experiments are currently ahead of schedule. However, the end product will be a mouse with 6 "alleles" (rtTA and cre transgenes, 2 floxed TSC1 alleles, and 2 MMP-9 null alleles), which will be very cumbersome and time consuming to generate. We would anticipate that these mice will not be ready to analyze until the end of the funding period.

Key Research Accomplishments

- Generation of a mouse model in which conditional targeting of the TSC1 gene in smooth muscle cells results in a reproducible phenotype (mortality at approximately 10 weeks of age).

- Alveolar duct enlargement in the lungs of these mice is a consistent finding.
- Formation of nodules in the lung of these mice is also a reproducible finding, although not as penetrant a phenotype as the mortality and the airspace enlargement.
- MMP-9 induction in the lungs of these mice may contribute to the airpspace enlargement (preliminary, gelatin zymography has been conducted on two conditional knockouts vs. two controls).
- Agreement in principle with Wyeth Pharmaceuticals to receive enough rapamycin to conduct a clinical trial in the mice (evaluating the effect of rapamycin on mortality, airspace enlargement, pulmonary nodule production, and MMP production)
- Establishment of lung fibroblast/smooth muscle cultures from the TSC1 conditional knockout mice (ongoing) for evaluation of MMP production and proliferation

Reportable Outcomes

The primary reportable outcome is the success of the conditional TSC1 knockout mouse (described above). These data will form the basis for an NIH R01 application to be submitted within the next month. Also, an abstract on this work was submitted and accepted for presentation at the American Thoracic Society international meeting to be held this May. The abstract is shown below:

Conditional Targeting of the TSC1 gene in Smooth Muscle as a Model of LAM

Yifu Fang*, David J. Kwiatkowski#, and J. Michael Shipley*
*Division of Pulmonary and Critical Care Medicine, Washington University School of Medicine, St. Louis, MO, 63110 and # Hematology Division, Brigham & Women's Hospital, Boston, MA 02115

Dysregulated proliferation of smooth muscle cells is a hallmark of LAM, which involves mutation of either the TSC1 (hamartin) or TSC2 (tuberin) tumor suppressor gene. TSC1 and TSC2 knockout mice die mid gestation, precluding analysis of their lungs as a model of LAM. To circumvent this issue, we developed transgenic mice for doxycycline-inducible expression of cre recombinase in smooth muscle and have used these to conditionally target the TSC1 gene. By RT-PCR, mice which express the reverse tetracycline transcriptional activator (rtTA) under the control of the smooth muscle alpha actin (SMA) promoter show significant rtTA expression in smooth muscle-containing tissues including the lung. These mice were bred to tetO-cre mice and ROSA26 reporter mice to assess doxycycline-inducible expression of active cre recombinase in the lung. Expression was seen in vascular smooth muscle, airway smooth muscle, and myofibroblasts. Bitransgenic SMA-rtTA/tetO-cre mice were bred to "floxed" TSC1 mice where targeting of the TSC1 gene was initiated by administration of doxycycline to the drinking water. Pregnant mothers were given doxycycline water continuously from conception to birth, and the offspring continued to receive doxycycline water throughout their lifespan. TSC1 flox/flox mice containing both the SMA-rtTA and tetO-cre transgenes that received continous doxycycline had a mean life span of 10 weeks (n=9). Hyperphosphorylation of ribosomal protein S6 kinase is seen in lung extracts, consistent with inactivation of hamartin function. Preliminary evidence suggests abnormal smooth muscle proliferation and airspace enlargement in the lungs of these

mice, two facets characteristic of human LAM. Thus, these mice appear to recapitulate important facets of the disease and will be useful in evaluating therapeutic interventions.

Conclusion

These studies should provide a viable model in which to study facets of tuberous sclerosis involving loss of function of TSC1 in smooth muscle, most notably the lung pathology which is also seen in lymphangioleiomyomatosis (LAM). The mice now provide a very useful tool in which to investigate the function of individual MMPs or other proteins in this pathological progression, and to evaluate relevant therapeutic interventions such as rapamycin.

References

N/A

Appendices

None